THE EFFECT OF NITROGEN, OXYGEN AND CARBON DIOXIDE IN PRODUCING THE DISTRESS SYNDROME IN TAPHIUS GLABRATUS (GASTROPODA, PULMONATA)

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The fresh-water snail, *Taphius glabratus* (*Australorbis* or *Planorbina* of authors, but see Harry, 1962), shows a special reaction, the distress syndrome, in concentrations of toxic substances which are intermediate between those concentrations allowing normal behavior and those producing sustained retraction (Harry and Aldrich, 1963). The most obvious symptom of the distress syndrome is the continued extension of the cephalopedal mass beyond the shell's aperture, without the attachment of the foot. The snail is apparently relaxed, or paralyzed, and in more severe cases there is gross sloughing of the cells in the distal parts of the tentacles, with basal tentacular swelling. Certain heavy metals, notably zinc, cadmium and copper, were found to evoke the distress syndrome in lower concentrations than any other of the 22 ions tested. Since the manifestation of distress is grossly the same regardless of the kind of ion which produces it, it is logical to assume that distress may be caused by a single physiological mechanism.

Yager and Harry (1964) compared the uptake of radioactive zinc, copper and cadmium by this snail. They found that under comparable conditions the patterns of uptake were similar for all three metals. Snails showing normal behavior often took up more of the metals than snails which were in distress. It was concluded that distress was dependent upon the concentration of the noxious ion in the external medium rather than on the amount of the ion which entered the snail. They further suggested that these substances may produce distress by impairing the permeability of the surface membranes, and that the impairment might be only partial, but sufficient to impede the required rate of passage of materials which must pass (*e.g.*, oxygen and waste materials) in order for the snail to maintain normal behavior.

The present study was made to explore the latter hypothesis. Specifically, we wished to determine whether distress could be produced by subjecting the snails to anaerobic conditions, or high concentrations of carbon dioxide or oxygen in the environment, and to determine what behavioral responses would result when the snails were exposed to various combinations of nitrogen, carbon dioxide and oxygen.

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Materials and Methods

All snails were from an inbred laboratory stock originating in Puerto Rico. They were maintained in 50-liter metal-framed aquaria containing Houston tap water. The water was filtered initially through charcoal, and continuous charcoal filtration was provided by external aquaria filters. A few grams of chemically pure calcium carbonate were added weekly, and aeration was continuously provided. Fresh lettuce constituted the only food. No substrate material or aquatic plants were present in the aquaria, but small sheets of polyethylene plastic were introduced, as this is preferentially used by the snails for egg deposition.

Snails selected for each experiment were placed for 30 minutes in about 200 ml. of distilled water, which was changed once. They were then blotted on paper towels and all surface water was removed with soft absorbent paper tissue. Each snail was weighed to the nearest milligram. The total range of weights for all snails used was 218 to 474 mg., averaging 330.9 mg. The weight range of snails used in any single experiment varied between 55 and 177 mg., averaging 110 mg. New snails were used for each experiment.

In some preliminary experiments the control snails showed distress in distilled water which had been prepared from tap water passed through a large ion-exchange apparatus providing the bulk distilled water for the laboratory, then redistilled in a glass container having a chromium-plated metal heating element exposed to the water. Analysis of this water by the dithizone method of Huff, as modified by Lakin *et al.* (1952), showed an appreciable amount of undetermined heavy metals was present. Water for the definitive experiments was therefore prepared by passing the bulk demineralized water of the laboratory through Amberlite MB-1 ion exchange resin (Mallinckrodt Co.). Following the "general test procedure," as described by Lakin *et al.*, we found that 100 ml. of this water did not produce a grossly detectable color change in 1.0 ml. of 0.0016% dithizone solution. This water did not distress the snails.

All glassware was chemically cleaned by washing it in Alconox, followed by rinsing in dilute hydrochloric acid and several rinses of the water demineralized with Amberlite.

Flowing system experiments. This series of experiments was designed to test the effect of regulated amounts of the several gases on the snails when waste products are minimized by diluting them. Pyrex Erlenmeyer flasks of 250-ml. capacity were used for test and control animals. One hundred ml. of the demineralized water were put in each flask, each of which was stoppered with a two-holed rubber stopper. One hole served as the exhaust vent, and through the other the gas was introduced through a small glass tube with its free end drawn out into a capillary tip, inserted well below the surface of the water. Plastic tubing connected the glass tubes to a manifold with brass valves which allowed the gas to be regulated in each flask. The manifold was connected to a water bath through which the gases were bubbled (250 ml. of distilled water) directly from the pressure tanks. All gases were obtained from a local supplier of industrial gases, and were guaranteed to be 99.5% pure, or better. Only enough gas was introduced into the experimental flasks to keep the water gently agitated. After the water had been flushed with the test gas for 30 minutes or longer, one snail was introduced into each flask. Five snails were used in each experiment, and each experiment was duplicated at least once. Controls consisted of two flasks similarly prepared but with air introduced from an aquarium air pump. All exposures were at room temperature (22–24° C.).

Snails were exposed for 24 hours to the experimental gas. Any snails which crawled above the water during the experiment were put down again by gently tilting the flask. Observations were made about hourly at the beginning and end of each experiment, and at occasional periods between.

At the end of the exposure period, the pH of the water was determined with hydrogen ion test papers immediately after the gas was turned off. The water and snail of each flask were then gently poured into individual beakers for observation during the following 24 hours. A small piece of lettuce was provided each snail during this recovery period.

Closed system experiments. These two series of experiments were designed to determine whether distress or sustained retraction could be induced by the accumulation of waste products of the snail with concomitant depletion of the oxygen supply. (1) *Boiled water experiment.* Pyrex reagent bottles of 500- and 60-ml. capacity and weighing bottles with ground glass stoppers of 30-ml. capacity were washed as described above. These were filled with demineralized water and set in a pan of water on a hot plate and boiled for 30 minutes. They were then cooled to room temperature, and the snails, previously kept for 30 minutes in demineralized water, were added to each bottle. A small amount of boiled, cooled water was added to fill the bottle, and the glass stopper inserted carefully, to exclude all bubbles of air. This was easily achieved in the 60- and 500-ml. bottles. In the 30-ml. weighing bottles, owing to the concave bottom of the stoppers, a large bubble was unavoidable. No attempt was made to seal the stoppers with wax or grease. Five bottles of 500-ml. capacity each contained one snail. Four bottles of 60-ml. capacity each contained one snail, four each contained two, and four each contained three snails. The weighing bottles each had one snail. (2) *Water with air experiment.* Stender dishes of 8-, 25- and 50-ml. capacity were cleaned as described. Two milliliters of demineralized water were put in each of the smaller dishes, 5 ml. in each of the medium-sized ones and 10 ml. in each of the larger ones. After one snail (previously conditioned for 30 minutes in demineralized water) was put into each dish, the glass tops of the dishes were sealed in place with Lubriseal (stopcock grease, A. H. Thomas Co.). Five dishes of each size were used in the experiment.

Results

The nature of the distress syndrome is such that it is only diagnosed with certainty after observing the snails over a period of several hours. This is particularly true if the distressed condition is mild. Snails attached but not crawling, or which crawled above the water, were considered normal in the present study. Those retracted just to the aperture of the shell or extended slightly beyond, but not attached, were considered to be in distress if they maintained this behavior for several hours. Only snails which remained retracted at least one-fourth whorl into the shell were diagnosed as "retracted," in the sense used by Harry and Aldrich (1963). In most cases the behavioral pattern exhibited at the end of 24 hours was assumed in one or a few hours after the experiment began, but in a few experiments the snail's reaction became more extreme during the experiment, passing from a normal to a distressed state, or from distress to sustained retraction.

In contrast to the ion-induced distress studied by Harry and Aldrich, where distressed snails were generally on the bottom of the bowl, most distressed snails in the present experiments floated at the water's surface.

The pH was 5.0 to 6.0 at the end of all experiments.

Flowing system experiments. The results of experiments in which gases were passed continuously through the experimental system are shown in Table I. The reaction of the snails was remarkably uniform in most experiments. In those few experiments in which more than one type of reaction was encountered, the number of snails showing a particular type is indicated as a prefix to the letter designating the reaction.

TABLE I

Response to various gas concentrations

% of gas			Reaction during	
N_2	CO_2	O_2	Experiment	Recovery
100	—	—	9N 1R	9N 1R
—		100	10N	10N
95	5	—	10D	10N
80	20		6D 4R	10R
	100	—	10R	10R
75	5	20	10N	10N
76	20	4	10N	8N 2R

N—Normal; D—Distressed; R—Sustained retraction.
Reactions as recorded at end of 24 hours' exposure and 24-hour recovery period.

The one snail which showed sustained retraction during the experimental period when exposed to nitrogen continued this throughout the recovery period, and may be discounted as an anomaly. All snails exposed to pure oxygen showed less tendency to crawl out and more active movement than did those of any other experiments.

The snails exposed to 5% carbon dioxide and 95% nitrogen tended to remain attached but motionless during the first 12 hours of the exposure. All had their foot well extended but not attached during the last few hours of the experiment, but there was no obvious tentacular damage. During the recovery period this mild distress persisted for a few hours, but by 24 hours all had become attached to the substrate and had fed on the lettuce.

Snails exposed to 20% carbon dioxide and 80% nitrogen all showed very typical distress in the first few hours of the experiment, with marked tentacular sloughing and basal swelling. By the end of the experiment four were retracted. During the recovery period the remainder became retracted, and all were obviously dead, with the water showing a bacterial turbidity.

In 100% carbon dioxide all snails retracted about one-fourth whorl into the shell within the first 30 minutes of the experiment, and this retraction was sustained during the entire experimental and recovery periods. Bleeding was plainly evident in the bronzing of the water, and the snails were evidently dead by the end of the recovery period.

The mixtures of gases containing both oxygen and carbon dioxide were actually mixtures of air and carbon dioxide, but the minute quantities of other gases theoretically present (chiefly argon) are calculated as nitrogen in Table 1. In those two experiments in which snails were exposed to 5% carbon dioxide, 20% oxygen and 75% nitrogen (CO_2 5%, air 95%), all snails were attached and frequently crawling during the exposure period, and all showed normal behavior and fed during the recovery period. The snails exposed to 20% carbon dioxide, 4% oxygen and 76% nitrogen (CO_2 20%, air 20%, nitrogen 60%) made repeated attempts to crawl out of the water, but none showed well-defined distress or sustained retraction during the experimental period. Most showed normal behavior during the recovery period, and the two which showed sustained retraction may be discounted as anomalous.

Closed system experiments. In the experiment in which snails were kept in closed bottles of boiled water without air, all were normal at the end of 60 hours. By 72 hours all were normal except for two in the 500-ml. bottles, one of which showed distress, while the other was retracted. All snails showed normal behavior in the experiments using Stender dishes throughout the 48 hours of the experimental period.

Discussion

Von Brand and co-workers (von Brand, 1955; von Brand *et al.*, 1950, 1955; Mehlman and von Brand, 1951; von Brand and Mehlman, 1953; Newton and von Brand, 1955) demonstrated that *Taphius glabratus* can withstand anoxic conditions for many hours. After establishing that 95% of these snails survived for 16 hours when exposed to pure nitrogen, while only 25% survived at 24 hours in their experimental system, they used 16 hours as the period of exposure to nitrogen in subsequent studies on the anaerobic metabolism of this snail.

In our experiments with approximately 100% nitrogen the results were similar to those of the workers cited. Evidently the majority of these snails can tolerate anoxia induced by nitrogen for at least 24 hours. Nitrogen was not as debilitating as carbon dioxide.

However, whereas our snails showed essentially normal behavior in nitrogen throughout the experimental period, von Brand *et al.* (1950; pp. 276 *ff.*) reported a reaction which is distinctly and typically distress, in all 18 species of prosobranchs and pulmonates (including *T. glabratus*) which they exposed to pure nitrogen: "The behavior of the snails under anaerobic conditions was quite characteristic. All extended maximally out of their shells and soon, at least within a few hours, became completely motionless. If not used for chemical determinations, the snails, after the end of the anoxic period, were placed into beakers containing aerated dechlorinated tap water. As long as they were fully extended, these recovered completely, resuming motion soon after restoration of aerobic conditions. If the anaerobic period lasted too long, on the contrary, the snails began to hemorrhage and finally retracted into their shells. This seemingly indicates that the above-mentioned lack of motion was not a complete paralysis. Snails which had retracted into their shells during the anaerobic period did not recover during a subsequent aerobic period. Whether, in all cases, they actually died during the anaerobic period, or died shortly thereafter, could not be determined."

Any of several factors may account for the snail's more extreme reaction to nitrogen which von Brand *et al.* found. Our experimental system was one in which the container was continually flushed with gas, which would have carried away and minimized the concentration of volatile waste products of the snail. The system used by the workers cited was a closed system of small volume, which allowed waste products to accumulate, and to be concentrated in the immediate vicinity of the snail. The fluid phase of our system consisted of 100 ml. of water, whereas they used only 2 ml. Thus, any non-volatile waste products would be in dilutions 50 times greater in our experiments than in theirs, and presumably the toxicity of such materials would be lessened by a comparable amount.

The amount of oxygen originally present in our system may have been greater than that in theirs, owing to the larger volume of water, but the initial period of flushing with the test gas, and continuous flushing throughout the experiment would minimize this. The nitrogen we used was taken directly from the tank, with no further treatment except bubbling it through a water trap, whereas that used by von Brand *et al.* was further purified by being passed over hot copper. It may be argued that sufficient oxygen was present in our gas to support normal activity of the snails, though impurities were probably less than 0.05% in the gas we used.

Probably the most significant difference in the two experimental systems was their use of tap water, which, though dechlorinated, might yet have contained enough copper to have produced distress. Such an amount of copper is often present in tap water, and can originate from the copper fixtures under sinks. The amount of copper is likely to be high in tap waters low in total dissolved solids (less than 100 parts per million).

Although Newton and von Brand (1955) have reported differences between strains of this snail in their ability to withstand anoxia, it is unlikely that this could account for the greater tolerance of the Puerto Rican strain we used than they found in two strains from South America. Their experimental system was such that distress and more severe reactions might have been produced by factors other than anoxia *per se*.

As von Brand (1946; p. 91; 1950; p. 273, *q.v.* for references to several earlier studies) has noted, "the fact that aquatic snails possess a certain tolerance toward the lack of oxygen has been known for some time." Our experiments merely indicate that this tolerance may be greater than previously realized in the case of *T. glabratus*.

The snails which we exposed to oxygen showed no marked disability, and were indeed more active and content to remain below the water line than in any other experiments. *T. glabratus* is thus not among those animals to which higher than normal concentrations of oxygen are toxic (von Brand, 1946; Fox and Taylor, 1955). Fox and Taylor (1955) kept the closely related snail, *Planorbarius corneus*, for over a month in flasks of water through which 100% oxygen was bubbled. Although they concluded that the snails slowly succumbed during that time, from 21% to 80% of the snails survived after 35 and 37 days, respectively, in the several experiments they reported. They also noted that *Planorbarius* lived well for over a month in water through which a gas mixture of 4% oxygen and 96% nitrogen was continuously bubbled.

Von Brand and Mehlman (1953) have shown that the oxygen consumption by *T. glabratus* is directly proportional to the partial pressure of the oxygen in the experimental gas to which they are exposed, in mixtures of oxygen and nitrogen in which the oxygen varies from 5% to 100%. The amount of oxygen consumed depended on the temperature, nutritional state and whether the snails had been exposed to anoxia.

There seem to be no previous studies on the effect of varying carbon dioxide tensions on fresh water snails. The severity of the disability produced by this gas in the absence of oxygen seems to be directly proportional to the partial pressure of the carbon dioxide, or, by implication, the tension of the carbon dioxide in the aqueous phase of the experimental system. While the exact limits of the concentrations of this gas which will allow normal behavior, produce distress or sustained retraction were not determined, it is significant that distress typical of that produced by inorganic ions was produced by intermediate concentrations of carbon dioxide, in the absence of oxygen.

In the presence of oxygen, partial pressures of 5% and 20% carbon dioxide were much less toxic. Whatever the mechanism of toxicity of carbon dioxide may be, its effect can be diminished if oxygen is available. This is the more remarkable since even a 20% partial pressure of carbon dioxide, which is about 666 times as concentrated as the partial pressure would normally be in nature, was only mildly debilitating to the snails in the presence of 4% oxygen, which is about one-fifth as concentrated as it would normally be in nature. In so far as the gases involved in respiration may be limiting factors to the ecological distribution of this snail, it would seem that the presence of carbon dioxide, rather than the absence of oxygen, is the significant factor. More specifically, the presence of elevated carbon dioxide concentrations in the complete absence of oxygen (as in some underground waters) may be a limiting factor in nature.

In the experiments in which the snails were in closed bottles of boiled water without air, and in the water with air in the Stender dishes, the dilution of waste products even in 2 ml. water and 8 ml. air (roughly comparable to the 2 ml. water and 15 ml. air in the Warburg flasks used by von Brand and co-workers) evidently would be sufficient to render such wastes innocuous. These experiments also support the view that the quality of water, or some factor other than oxygen depletion and waste accumulation produced the distress reported by von Brand *et al.* in the anoxic experiments they devised.

Although the results reported above shed no light directly on the mechanism responsible for producing distress, they are compatible with the hypothesis that the failure of passage of materials at critical rates through surface membranes may be one factor in producing this phenomenon. It is generally assumed that the integument of fresh-water snails (Zaaijer and Wolverkamp, 1958; p. 67) as well as other organisms (Jacobs, 1920a, 1920b; Brandt, 1945) is very permeable to gases, but this does not exclude the possibility that there may be factors, including the materials previously shown to produce distress, which can greatly alter the permeability of the membranes to gases. Brandt (1945; p. 32 *ff.*) states that carbon dioxide enters cells more rapidly than mineral acids, oxygen, nitrogen or other gases. He cites several references of more extensive studies on this aspect of the subject, but concludes, "it is at present unknown in what way carbon dioxide affects the state of the protoplasm and its function, except for its probable effect on pH." Jacobs (1920a, 1920b) had earlier argued that carbon dioxide probably has some specific effect on biological phenomena, quite apart from its ability to influence the pH.

Nothing is known of the ability of the blood of *Taphius glabratus* to transport carbon dioxide and oxygen. Zaaijer and Wolverkamp (1958) studied the hemoglobin-oxygen equilibrium in the blood of *Panorbarius corneus*, finding quite normal curves in the absence of carbon dioxide, when only temperature and the partial pressure of oxygen were varied. But when they introduced the additional factor of carbon dioxide and varied the pH in their pooled *in vitro* samples, they encountered "highly irregular and unpredictable" results, often including a negative Bohr effect when the pH was reduced below 8.0. They concluded that the blood buffering substances, proteins and salts of these snails may be variable. Targett (1963) recently showed that the blood proteins of *Taphius glabratus* may vary quantitatively and qualitatively, for causes yet unknown.

Zaaijer and Wolverkamp consider only the concentration of carbon dioxide in the external environment in seeking an explanation for its effect on hemoglobin, and they overlooked the possibility that the amount of carbon dioxide produced by the snail may be significant. Von Brand *et al.* realized the importance of the snail's own metabolism as a source of carbon dioxide, but point out in several papers that it is impossible at present to measure the internal concentration of this gas, owing to the interference produced by the snail's shell. To this we may add that the ubiquitous calcareous granules of the snail's connective tissue might also contribute to this difficulty, even if carbon dioxide production of snails with shell removed were to be studied. In any event, among numerous papers on snail respiration studies, no data on carbon dioxide production in aerobic conditions have been published, but its production in anaerobic conditions have been reported by Mehlman and von Brand (1951) and Newton and von Brand (1955).

Summary

1. *Taphius glabratus* shows no abnormal reaction to anoxia induced by nitrogen during 24 hours' exposure. It also shows normal behavior in pure oxygen for that length of time.

2. In the absence of oxygen, 5% carbon dioxide produces mild distress, from which snails recover; 20% carbon dioxide evokes severe destress, from which snails did not recover; and 100% carbon dioxide resulted in immediate retraction and death.

3. In the presence of even smaller than normal amounts of oxygen, 5% and 20% carbon dioxide produced no deleterious effects on the snail.

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